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BIOLOGICAL AND PHYSICAL PROPERTIES OF A HUMAN γM-CRYOGLOBULIN AND ITS MONOMER SUBUNIT

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SUMMARY

A patient with Waldenstrom's macroglobulinaemia was found to have a serum cryoprecipitate which consisted entirely of a γMK-macroglobulin. This protein had no detectable antibody activity against a variety of other serum components and fixed only minimal amounts of complement over temperatures ranging from 4°C to 37°C. Precipitation of the cryoglobulin began at about 30°C and was complete at temperatures below 20°C. In contrast to other macroglobulins that have been reported, cryoprecipitability persisted after the protein was dissociated into its monomer subunits. Isolated subunits formed cryoprecipitates as did the hybrid macroglobulin formed from equal parts of such subunits and the subunits of a non-cryoprecipitating macroglobulin.

Carbohydrate content of the cryoglobulin was 12·81%, a value similar to γM molecules in general. Enzymatic removal of a portion of the carbohydrate caused the protein to become insoluble at even higher temperatures.

A single type of intermolecular binding responsible for cryoprecipitation could not be defined. Addition of salt or non-polar solvent, or change in pH had little effect on cryoprecipitability at 4°C, but all of these measures did cause an increased solubility at 24°C, a transitional temperature for cryoprecipitability. Addition of a variety of serum proteins and glycine also served to increase protein solubility. These data suggest that cryoprecipitation may involve both hydrophobic and electrostatic bonds. Temperature induced changes in molecular configuration might also indirectly influence cryoprecipitation by altering the binding site.

INTRODUCTION

Cryoglobulins appear to fall within two general categories. In one group, two immunoglobulin classes are present, one acting as the antibody directed against the other as the antigen (Meltzer & Franklin, 1966; Lo Spalutto *et al.*, 1962). The other type of cryo-

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globulin includes those which usually have no antibody activity but exhibit increased intermolecular attraction at low temperatures. A single homogeneous immunoglobulin component is found in this category (Meltzer & Franklin, 1966).

In this report, we present data on a serum γM-cryoglobulin from a patient with Waldenstrom's macroglobulinaemia which belongs to the second category. These observations deal with the nature of the intermolecular attractive forces, complement fixing ability and some comparative aspects of the *in vivo* and *in vitro* behaviour of this cryoglobulin and its monomer subunits.

CASE REPORT

L.E., a 60-year-old white male auto mechanic, was well until May, 1965, when he was found to be anaemic. A serum cryoprecipitate was first seen at that time. Generalized lymphadenopathy, weight loss, anorexia, and fatigue began in 1966. He was hospitalized in February, 1968, at which time he had multiple enlarged non-tender lymph nodes in the cervical, supraclavicular, axillary and inguinal areas. His spleen was palpable 2 cm below the left costal margin. Laboratory results at that time were: haematocrit 37%, haemoglobin 10·9 g%, white blood cell count 8300/mm^3 with a normal differential count, blood urea nitrogen 16 mg%, creatinine 1·3 mg%, uric acid 5·1 mg%, serum protein 9·4 gm%, albumin 2·8 gm%, γ-globulins 4·4 gm%. A urinalysis and a urine Bence-Jones test were negative. Serum immunoglobulin levels were γA 47 mg%, γG 120 mg% and γM 3700 mg%. Serum viscosity measured in an Ostwald viscosimeter at 37°C and 24°C were 150 and 190 seconds compared to values of 82 and 85 seconds for a normal serum. The serum complement level was 40 CH_{50} units per ml (normal range 40–50 units). Rheumatoid factor and anti-nuclear factor tests were negative. A massive cryoprecipitate was present in serum cooled to 4°C which caused the serum to take on the appearance of freshly clotted plasma. Bone marrow examination revealed a lymphocytic infiltration compatible with a diagnosis of Waldenstrom's macroglobulinaemia.

There was no history of vascular insufficiency, purpura, or cold sensitivity. Recurrent or chronic infections have not been a problem.

After a course of cyclophosphamide and radiation therapy, lymphadenopathy decreased. The patient has returned to work and when examined in January, 1970, was asymptomatic except for weakness. The cryoglobulin content of his serum and its precipitability were unchanged. The serum complement level was 55 CH_{50} units per ml.

MATERIALS AND METHODS

Isolation of cryoglobulin

Blood from the patient was maintained at a temperature of 37°C until clot retraction had occurred. The serum was removed and then cooled to 4°C overnight. The precipitate was removed by centrifugation and was then dissolved in phosphate buffered saline (PBS) at pH 7·0 which had been prewarmed to 37°C. The solution was then cooled in an ice bath and the precipitate which formed was again removed by centrifugation. This procedure was repeated 15 times. Cryoglobulin prepared in this manner was used in most of the experiments reported below. The final preparation was a pure γMK-cryoglobulin by criteria of immunoelectrophoresis, immunodiffusion and analytical ultracentrifugation.

Isolation of non-cryoprecipitating γM-globulins

These proteins were obtained from the serum of a patient with Waldenstrom's macrobulinaemia (Simp) and a pool of 19S rheumatoid factor containing sera. Gamma-M (Simp) was isolated from serum by starch block electrophoresis and Sephadex G-200 chromatography. The γM rheumatoid factor was isolated by the method of Butler & Vaughan (1965) by absorbtion of rheumatoid factor on a γ-G globulin containing immunoadsorbent with subsequent elution with a glycine hydrochloride buffer, pH 2·8. The eluted rheumatoid factor was neutralized with 0·5 N NaOH, concentrated by vacuum dialysis, and fractionated by centrifugation for 16 hr in a Spinco model L ultracentrifuge using 10–40% discontinuous sucrose gradient. The fractions containing 19S rheumatoid factor were dialysed against PBS, pH 7·0 overnight. This fraction contained only a γ-M type rheumatoid factor when tested on immunodiffusion in 1% agarose against antisera specific for human γ-M, γ-G and γ-A.

Crosslinking of proteins

Crosslinking of cryoglobulin γ-M as well as rheumatoid factor γ-M globulin pooled normal γ-G globulin (Cohn fraction II) and bovine serum albumin (BSA) in equimolar concentrations was performed by the method of Waldesbuhl *et al.* (1970) using a 6M solution of N,N-dimethylformamide with incubation at 37°C for 10 min.

Equimolar concentrations of these same proteins were also cross-linked by glutaraldehyde using a 10:1 ratio of glutaraldehyde to protein (Avrameas & Ternynck, 1969). The incubation time used for cross-linking was 60 min at 37°C. The cross-linked proteins were extensively dialysed against several changes of PBS followed by barbital buffer, pH 7·3.

Formation of soluble protein aggregates by N,N-dimethylformamide cross-linking was demonstrated by turbidimetric readings at 450 μ in a Beckman spectrophotometer. The optical density readings at this wavelength were identical for the γ-M cryoglobulin and γ-M rheumatoid factor (at equal concentrations) suggesting formation of aggregates of equal size. Formation of polymers by glutaraldehyde was demonstrated by ultracentrifugal sedimentation studies in linear sucrose density gradients (10–50%).

Ultracentrifugation

A model E Beckman-Spinco ultracentrifuge was used to determine sedimentation velocity. All runs were performed at 59,780 rev/min at 37°C using schlieren optics. Sedimentation coefficients were corrected to 20°C in water, but no corrections were made for protein concentration.

Carbohydrate determinations

The hexose content of the cryoglobulin was determined by the orcinol method (Kabat & Mayer, 1961). Fucose was measured by the Gyorky & Houck (1965) modification of the Dische-Shettles procedure (1948). A modification of the Elson-Morgan procedure (Boas, 1953) was used to assay hexosamine content after the sample had been hydrolysed with 4 N HCl at 100°C for 6 hr and isolated with Dowex 50. The thiobarbituric acid assay for neuraminic acid (Aminoff, 1961) was used after the bound neuraminic acid was released by acid hydrolysis (0·1 N H_2SO_4, 80°C, 1 hr).

Glycosidases

A portion of the carbohydrate was removed with glycosidases in the following manner.

Neuraminidase, 43 μ per mg* of cryoglobulin, was allowed to react for 48 hr at 37°C using an 0·005 M sodium acetate-acetic buffer (pH 5·5 with 0·9 g NaCl and 0·1 g $CaCl_2$ per litre. The cryoglobulin was then dialysed against a 0·1 M citrate, pH 4·5, buffer. B-Glucosaminidase, β-galactosidase and β-mannosidase isolated from jack bean meal by a procedure described in an earlier publication (Andersen, 1969) were added to the cryoglobulin so that their activities were 3, 4, and 3 u† per mg of protein respectively. The sample was incubated at 37°C for 10 days.

After a glycosidase treatment, the cryoglobulin was dialysed with many changes of distilled water to remove the free sugars. The dialysis baths were pooled, desalted with Amberlite MB-2 and brought to dryness. The free sugars from the dialysis bath were separated by descending paper chromatography with a pyridine, *n*-butanol, water (4:6:3) solvent system. A silver nitrate reducing sugar stain identified the sugars released by the enzymes using known sugar controls. The cryoglobulin was dialysed against PBS prior to evaluating cryoprecipitability.

Reduction and Alkylation

Reduction was accomplished with 0·1 M 2-mercaptoethanol in PBS. The reaction was carried out at 37°C for 1 hr. Those samples which were not alkylated were dialysed against PBS with 10 min bath changes for 2 hr then continued overnight dialysis. Alkylation was performed with a five-fold excess of 3-times crystallized iodoacetamide. A similar dialysis procedure was used to remove excess iodoacetamide.

Complement studies

Normal human serum was used as a source of complement in all studies on complement fixation. It was absorbed three times at 4°C with washed sheep red cells to remove anti-Forsmann antibodies. The complement titre was determined by the method of Kabat & Mayer (1961) using sheep red cells optimally sensitized with rabbit γM haemolytic antibody. The volume of all reagents used was reduced to 40% but all results are expressed in terms of the full volume system. Sheep red cells and haemolytic amboceptor were supplied by local commercial sources.‡

The method of complement fixation used in this study was modified from that of Kabat & Mayer (1961). Six CH_{50} units of complement were added to the assay tubes containing the cryoglobulin and other reactants in a final volume of 2·6 ml. All materials used in the complement fixation procedure were kept at the indicated temperature during fixation. The amount of complement remaining after fixation was determined by incubation of aliquots of the fixation mixture for one hour at 37°C using sheep red cells optimally sensitized with γM amboceptor. Such aliquots were warmed to 37°C immediately prior to this step.

RESULTS

Character of the cryoprecipitate

In the isolation procedure, it was discovered that after three cycles of washing the resolubilized precipitate consisted primarily of a γM-globulin bearing kappa light chains as

* Hoechst Pharmaceutical Company (Behringwerke).

† Enzyme units are described in an earlier paper (Andersen, 1969).

‡ Markham Laboratories, Chicago, Illinois, U.S.A.

demonstrated by Ouchterlony double diffusion analysis with specific antisera. Small amounts of C3 and fibrinogen could be detected. Additional washing of precipitate resulted in a preparation that contained only γMK-globulins indicating a cryoglobulin homogeneous in composition.

The isolated cryoglobulin was white and amorphous in character except when it was formed in high concentration at which time much of it took a clear gelatinous form. The gel could be dissolved by rewarming to 37°C, but it usually took longer to dissolve than the amorphous form. The dissolved gel was capable of reforming either the gel or the amorphous precipitate on cooling.

Fig. 1 shows the amount of cryoprecipitation which occurred after the isolated cryoglobulin had been cooled to various temperatures for 90 min. The cryoglobulin, at a

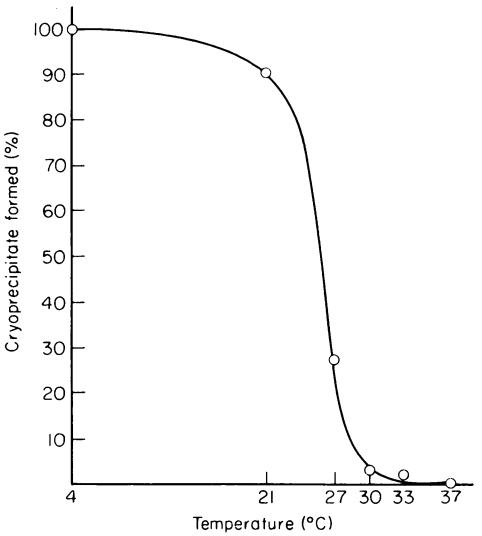

FIG. 1. Effect of temperature on the precipitability of the cryoglobulin.

concentration of 8·7 mg per ml, was in a phosphate saline pH buffer. It was apparent that under these conditions, cryoprecipitation began to occur at about 30°C and was essentially complete below 21°C.

Sedimentation coefficient

Ultracentrifugation in a Beckman-Spinco model E ultracentrifuge at 59,780 rev/min showed that the major component of the isolated cryoglobulin has a sedimentation coefficient (S_{20w}) of 15·4S (protein concentration 8–10 mg per ml). There was also a minor component (<5%) with a higher sedimentation coefficient ($S_{20w}=22·8$) which probably was a γ-M cryoglobulin aggregate. These sedimentation coefficient values would be somewhat higher if corrections for protein concentrate had been made.

Carbohydrate composition

The results of quantitative carbohydrate determinations done on the cryoglobulin, as well as on a γ-M globulin preparation isolated from another patient with Waldenstrom's macroglobulinaemia but which lacked cryoprecipitability, are shown in Table 1. The two

preparations had similar amounts of carbohydrate. These values agree rather closely with those reported for the 'high carbohydrate' γM-globulins by Davie & Osterland (1968).

Reduction and Alkylation

The cryoglobulin, after reduction and alkylation, retained its ability to precipitate in the cold as did the cryoglobulin treated only with iodoacetamide. In order to determine the size of the molecule which retained cryoprecipitability washed cryoprecipitate from the reduced and alkylated sample was applied to Sephadex G-200 column and chromatographed at 37°C. Two peaks appeared, the first one at the void volume. The second and larger peak contained most of the cryoprecipitating material. The sedimentation coefficient (S_{20w}) of the second peak was 6·3S, as determined in the model E Beckman-Spinco ultracentrifuge at 59,780 rev/min and 37°C. This isolated γM subunit that retained cryoprecipitability reacted with a specific anti γM serum.

It also was possible to show that hybrids formed from subunits of cryoglobulin and non-cryoglobulin γM retained cryoprecipitability. In this experiment the cryoglobulin, which was a γMK-globulin, was mixed with a non-cryoglobulin protein of γMλ type from another patient (Simp) with Waldenstrom's macroglobulinaemia. The γMK/γMλ ratio was

TABLE 1. Percent carbohydrate

	Fucose	Neuraminic acid	Hexose	Hexosamine	Total
Cryoglobulin (γM)	0·88	1·25	5·92	4·76	12·81
Non-cryoglobulin (γM-Simp)	0·79	1·34	4·82	4·24	11·19

1·0 (2·3 mg per ml of each). The mixture was reduced with mercaptoethanol and dialysed for 18 hr. Recovery of cryoprecipitability after removing the reducing agent was excellent. The sample was washed by repeated cycling through the cryoprecipitation stage as it was described in the initial preparation of the cryoglobulin. Ouchterlony double diffusion analysis with specific anti-K and anti-λ sera showed that both types of L-chains were present. Controls included mixing both γMK and γMλ proteins but omitting the reducing agent. No lambda chains were detected in the washed cryoprecipitate showing that simple mixing of these two γM-globulins was not responsible for coprecipitation of the non-cryoglobulin. These observations indicate that the γM subunits formed hybrid γM polymers which retained cryoprecipitability. If non-cryoprecipitating hybrids had also been formed, they would not have been recovered since they would have been lost in the washing procedure.

Sulphhydryl blockers

In an experiment to evaluate the role of free sulphhydryl groups in cryoprecipitation, the sulphhydryl blockers *n*-ethyl maleimide and *p*-chloro mercuribenzoic acid were added in ten-fold molar excess to the cryoglobulin in a PBS buffer. Both blockers failed to alter the cryoprecipitability of this protein excluding the possibility that free sulphhydryl groups are involved in the reaction.

Effects of salt concentration and pH on cryoprecipitability

In order to evaluate the role of electrostatic factors in the formation of the intermolecular bonds of cryoprecipitates, salt concentration and pH were independently varied.

Using a cryoglobulin concentration of 1·5 mg/ml in a 0·007 phosphate pH 5·2 buffer, varying amounts of NaCl were added, and the amount of cryoprecipitation was observed ut 4°C, 24°C, and 37°C. It can be seen from Fig. 2 that at 4°C there is essentially no decrease in cryoprecipitability over a wide range of salt concentration. At 24°C, there was some inhibition of cryoprecipitation at higher salt concentration. At this temperature, however, cryoprecipitation would be most susceptible to change since it is in the middle of the

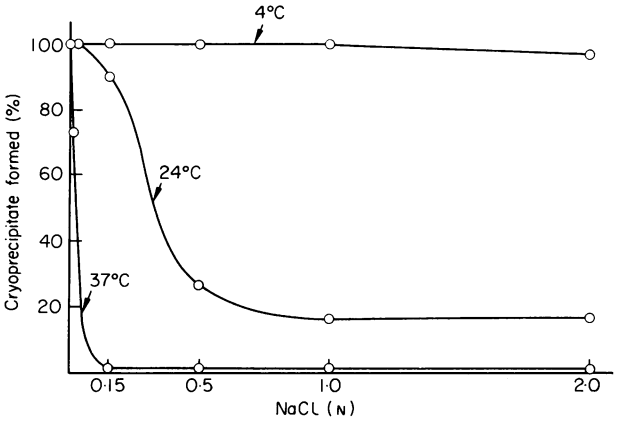

FIG. 2. Precipitability of the cryoglobulin at varying salt concentrations and temperatures.

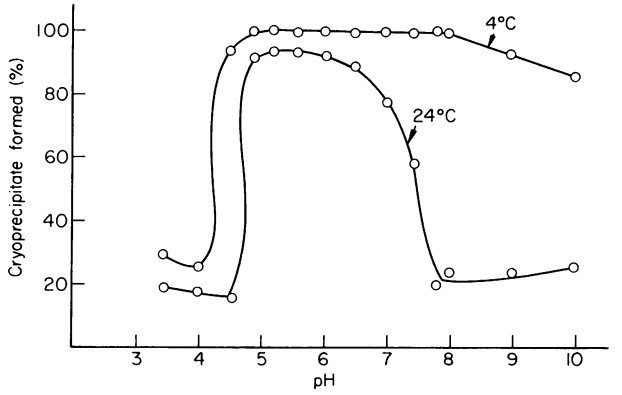

FIG. 3. Cryoprecipitability at varying pHs and temperatures.

transition zone (Fig. 1). The precipitation at 37°C in low salt concentrations is a mainifestation of the well-known 'euglobulin' behaviour of γM-globulins. The set of curves indicate that except at transitional temperatures, increasing salt concentrations have no inhibitory effect on cryoprecipitability.

The effect of pH on cryoprecipitability was determined using 10 mg/ml of the cryoglobulin in 0·007 M citrate or phosphate buffer containing 0·15 M NaCl. The pHs ranged from 3·5 to 10. The samples were allowed to cool and the fraction which failed to precipitate was

determined by measuring the optical-density of the supernatant solution at 280 mμ. The samples were then allowed to warm at 24°C, and the supernatant solutions were read again at OD 280 mμ. From the data in Fig. 3, it can be seen that at 4°C, over a wide pH range there is no loss of cryoprecipitability. At the extremes of the pH range (<4·5:>9) there is some loss of cryoprecipitability. Since it is possible that intramolecular changes (i.e.—denaturation) are occurring at these very high and low ranges, we cannot be certain whether the loss of cryoprecipitability is due to inter- or intramolecular events. Even at the transitional temperature (24°C) there is a fairly broad plateau of cryoprecipitability. These observations, coupled with the minimal effect of increasing salt concentration on cryoprecipitability, suggest that electrostatic forces do not play a dominant role in the cryoprecipitation of this protein.

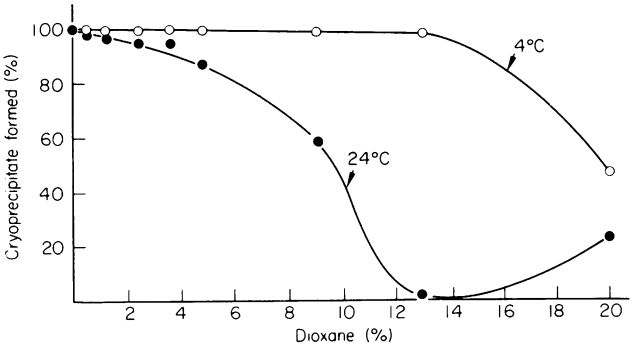

FIG. 4. Effect of varying concentrations of dioxane on cryoprecipitability.

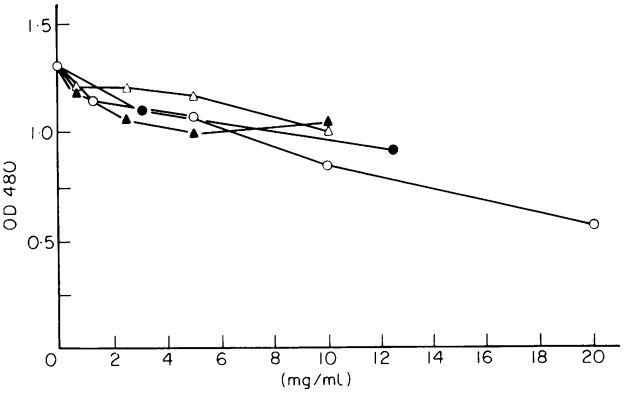

FIG. 5. The effect of several serum proteins and glycine on cryoprecipitability at 25°C. △, IgG; ▲, IgM; ●, Glycine; ○, Albumin.

Effect of non-polar solvents

If hydrophobic bonds are a major force in the intermolecular bonds of cryoprecipitation, then procedures that would weaken this type of bond would prevent cryoprecipitation. Non-polar solvents should effectively eliminate hydrophobic bonds. In this experiment, 1, 4 dioxane was added in gradually increasing amounts to 10 mg of cryoglobulin per ml in 0·007 M phosphate pH 5·2 with 0·15 M NaCl. The effect of this non-polar solvent on cryopre-

cipitability at 4°C and 24°C is seen in Fig. 4. At 4°C, there was essentially no effect with dioxane concentrations up to 13%. At 20% dioxane, cryoprecipitability was reduced to 50%. At that concentration, however, the dioxane probably was causing protein denaturation, since irreversible precipitation was found. At 24°C, the transition zone for cryoprecipitability, the effect of dioxane was greater in inhibiting cryoprecipitation. These data indicate that hydrophobic bonding might account for some of the cryoprecipitability.

TABLE 2. Complement levels in fresh serum at varying temperatures

Serum	0 hr (37°C)	1½ hr (37°C)	1½ hr (30°C)	3 hr (24°C)	18 hr (4°C)
Normal Serum	38	32	31	33	32
γM-cryoglobulin serum	55	40	42	50	40

Complement levels (CH_{50} units/ml) of the patient's own serum were determined after incubation of serum at different temperatures for varying periods of time. Serum obtained from fresh blood clotted for 30 min at 37°C is considered to be at 0 hr.

TABLE 3. Complement fixation by isolated proteins

Incubation temp. and time	Protein (μg)	γM-Cryo		γM-RF		γG	BSA
		Cross-linked	Native	Cross-linked	Native	Cross-linked	Cross-linked
37°C; 90 min	500	0·1	0·3	2·8	—	>3·5	0
	50	0·3	—	1·1	0·6	>3·5	—
	5	—	—	—	—	0·9	—
	1	—	—	—	—	0	—
4°C; 16 hr	500	0	0	1·1	—	4·8	0
	50	0	—	0	0	4·3	—

Complement fixation by different amounts of γM-cryoglobulin (Cryo) and γM-rheumatoid factor (RF) cross-linked by dimethylformamide and in native form, cross-linked bovine serum albumin (BSA) and cross-linked human γG (Cohn, Fraction II).

Number of units fixed out of 6 CH_{50} units added is recorded after correction for decay of complement in control tubes (2·5 CH_{50} units at 37°C and 1·2 CH_{50} units at 4°C).

(–) indicates test not done.

However, since dioxane failed to cause reversible inhibition of cryoprecipitability at lower temperatures (4°C) it might be argued that additional intermolecular attractive forces exist apart from hydrophobic bonds.

The effect of glycosidases on cryoglobulin

Glycosidase treatment caused a loss of approximately a fourth of the cryoglobulin carbohydrate. The glycosidase-treated cryoglobulin was compared to control samples of cryoglobulins which had been handled in a similar fashion except for omitting the enzymes.

The control proteins had thermal solubility properties similar to samples that had been described earlier, but the glucosidase-treated cryoglobulins were less soluble. The precipitate was insoluble after warming the sample to 37°C for 18 hr. At 45°C, some of the precipitate went into solution, but complete solubilization did not occur until the temperature was raised to 52°C.

Effects of other proteins and glycine on cryoprecipitation

In the course of isolating and characterizing the cryoglobulin, we observed that cryoprecipitation was inhibited by a variety of proteins In order to provide a more accurate measure of this phenomenon, nephelometric determinations of cryoprecipitation were made at 25°C by measuring optical density at 480 mμ. Human serum albumin, normal pooled human γG-globulin, a non-cryoprecipitating γM-globulin from a patient with Waldenstrom's macroglobulinaemia (Simp), and glycine were tested for their ability to inhibit cryoprecipitation. Cryoglobulin in a concentration of 5 mg/ml in PBS (pH 7·0) was combined with varying concentrations of the above substances at 37°C and allowed to cool at 25°C. After remaining at 25°C for 30 min, the optical densities were determined. It can be seen in Fig. 5 after appropriate blank corrections were made, that turbidity decreased in all cases. Since no large particles or settling of precipitate were observed to account for lessening of turbidity, the changes in optical density must have been due to decrease in cryoprecipitability. In many of the tubes, there was a visible clearing of cryoprecipitation.

Complement fixation studies

Serum was separated from blood freshly obtained from the patient and kept at 37°C. When tested one-half hour after venesection, it had a level of 55 CH_{50} units per ml, a value just above the normal range. If the serum was incubated at 37°C, 30°C, 24°C, or at 0°C for varying periods of time, some loss of complement resulted at all temperatures (Table 2). But this loss was of the same magnitude as the loss that occurred in a normal serum. No additional fixation of complement occurred at lower temperatures (24°C or at 0°C) where cryoprecipitation was noted.

Purified fractions of native γM-cryoglobulin were tested next for their ability to fix complement. Large amounts of γM cryoglobulin (500 μg) fixed at most insignificant amounts of complement at 37°C and none at 4°C.

Gamma M-cryoglobulin was cross-linked by dimethylformamide to assess, by means other than cryoprecipitation, the role of protein aggregation in complement fixation. After cross-linking, the γM-cryoglobulin fixed barely detectable amounts of complement. A control protein, BSA, after cross-linking, gave similar results. In comparison, cross-linked γG-globulin and another γM-globulin (rheumatoid factor) fixed considerable amounts of complement (Table 3). Native γM rheumatoid factor that had not been cross-linked did, however, fix a small amount of complement. This may have been due to minimal aggregation occurring during its preparation or interaction with γG-globulin in the complement serum.

DISCUSSION

The protein described in this report falls into the category of a homogeneous cryoprecipitate. It consists entirely of γMK-globulin. Had it possessed antibody against a deter-

minant on light chains, the precipitate would have consisted of several types of immunoglobulins, all with single light chain specificity. Since no molecules of γG or γA were found, this possibility can be excluded. If it possessed antibody activity with anti-μ chain specificity, both types of light chains, lambda as well as kappa, should have been found in the cryoprecipitate. It is, of course, theoretically possible that the cryoglobulin contains an antibody with specificity against some unique antigen present only on that cryoglobulin itself. Such a situation would indeed cause a homogeneous cryoprecipitate. This remote possibility has not been excluded but appears rather unlikely to these investigators.

Other studies (Meltzer & Franklin, 1966; Curtain, Baumgarten & Pye, 1965) have shown that reduction and alkylation which caused depolymerization of native γM molecules to subunits results in loss of cryoprecipitability. This is at variance with results obtained with our cryoglobulin which showed persistent cryoprecipitability of the subunits. In one of the reports (Curtain *et al.*, 1965) the cryoglobulin was of the antigen–antibody or heterogeneous type. In the other report (Meltzer & Franklin, 1966), it was not clear which type was involved. The response to depolymerization of a γM antibody that requires interaction with its antigen to cryoprecipitate might be quite different from a homogeneous cryoprecipitate in which other intermolecular bonds are invoked by cold. The ability of our subunits to cryoprecipitate suggests that direct interaction of subunits (either isolated or on different intact γMs), rather than intramolecular subunit interaction, is necessary for cryoprecipitation.

Meltzer & Franklin (1966) demonstrated that when repolymerization of γM-cryoglobulin subunits occurred in the presence of subunits from non-cryoprecipitable γM-globulins, cryoprecipitability was lost. We were able to form γM hybrids from our γM-cryoglobulin and another non-cryoprecipitating γM which retained cryoprecipitability. This result is in keeping with the finding of cryoprecipitability retained by the isolated subunits themselves and further supports the concept that cryoprecipitation is due to intermolecular binding between sites present on γM subunits.

To explain the cryoprecipitation exhibited by our protein and other examples of homogeneous cryoprecipitates, one must consider three general types of intermolecular non-covalent bonds, namely, hydrophobic, electrostatic, and hydrogen bonding. The surface of homogeneous cryoglobulins may have a favourable distribution of amino acids which would permit one or several of these types of non-covalent bonding to occur.

The carbohydrate moiety of proteins has been considered by most to increase solubility by hydrogen bond formation between the hydroxyl groups on the carbohydrate and neighbouring water molecules. The carbohydrate of our cryoglobulin appeared quantitatively and qualitatively normal. If the carbohydrate had been abnormally low, it could have contributed to the insolubility of the cryoglobulin. We also considered the possibility that intermolecular hydrogen bonding involving carbohydrate hydroxyl groups on cryoglobulin molecules could be responsible for cryoprecipitation even though hydrogen bonding in an aqueous environment is generally very weak because of preferential hydrogen bonding with water. This idea was excluded by the failure to decrease cryoprecipitation after removal of some of the carbohydrate groups with glycosidase treatment.

Electrostatic bonding may play some part in the intermolecular attraction of our cryoglobulin, but it appears to be of insufficient strength to be the only factor involved. In addition, it is unlikely that electrostatic bonds would explain the temperature dependence of this reaction since electrostatic reactions tend to be athermic (Kauzmann, 1959).

Hydrophobic bonding has been invoked as a mechanism responsible for protein-protein

interactions. One excellent example is the observation of Murayama (1964) on sickling of haemoglobin S. He demonstrated that the reaction was endothermic by showing loss of sickling at lower temperatures and proved that hydrophobic bonds were responsible by unsickling the haemoglobin S in a non-polar environment.

In one of the few studies attempting to evaluate the nature of the intermolecular binding forces in homogeneous or 'non-antigen-antibody' cryoprecipitates, Saha *et al.* (1968) examined a γGl-cryoglobulin. They compared the amino acid analysis of the patient's cryoglobulin with his normal γ-G globulins and found that the cryoglobulin had significantly increased amounts of hydrophobic amino acids. In ultracentrifugation studies, they also observed a smaller frictional ratio for the cryoglobulin which indicated a smaller and more spherical shape than normal γ-G globulins. This change in shape could be due to a greater number of hydrophobic amino acids. However, when these authors compared the amino acid content of their cryoglobulin to a non-cryoprecipitating γ-G1 globulin, there was no significant difference in the number of hydrophobic amino acids. Thus, they were not able to exclude the possibility that the amino acid values and frictional ratios observed for their cryoglobulin, although different for γ-G globulins in general, fell within the range of values for some non-cryoprecipitating γ-G1 globulins. Indeed, more detailed studies of actual amino acid sequences in a number of individual globulins will be required before it will be possible to definitively assign cryoprecipitability to such a mechanism.

In our experiments with our cryoglobulin, we have been able to show that the addition of non-polar solvents caused a decrease in cryoprecipitability at transitional temperatures but not at 4°C. This minimal effect suggests that hydrophobic bonds may contribute to the intermolecular reaction but are not likely to explain it entirely. This is not surprising since hydrophobic reactions are not exothermic in nature (Kauzmann, 1959).

The mechanism of cryoprecipitation of our cryoglobulin cannot be clearly defined at the present time. In addition to the hydrophobic and electrostatic forces, temperature-dependent intramolecular changes may influence the reaction by altering the intermolecular binding sites. Intramolecular hydrophobic bonds, for example, might be weakened at low temperatures with a subsequent unfolding of the molecule and exposure of the intermolecular binding sites. The ultimate nature of these reactions, however, can only be firmly established by a greater understanding of the tertiary conformation of the cryoglobulin molecule.

Recently, two classes of γM-globulins have been found in sera from human as well as other species (MacKenzie *et al.*, 1969; Hoyer *et al.*, 1968; Plotz, Colten and Talal, 1968; Tesar *et al.*, 1969; Glovsky & Fudenberg, 1969). One had the capacity to fix significant amounts of the complement, while the other did not. We suggest that the cryoglobulin reported here belongs to the class of macroglobulins having no capacity to fix complement. Other homogeneous γM-cryoglobulins with complement-fixing ability were not available for comparison, but after aggregation by means other than cold, that is, treatment with dimethylformamide or glutaraldehyde, our cryoglobulin was shown to activate much less complement than did equal amounts of a purified preparation of γM rheumatoid factor.

Our patient's lack of clinical complications from cryoglobulinaemia becomes more understandable in view of some of our experimental observations. Consumption of complement in his own serum *in vitro* was absent at body temperature as well as at temperatures at which cryoprecipitation occurred. Another factor in reducing the pathogenicity of our cryoglobulin is the ability of serum proteins to inhibit cryoprecipitation. This probably prevented the formation of cryoglobulin aggregates that would otherwise develop in the

peripheral circulation where temperatures may fall below those which were shown to produce *in vitro* precipitation.

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REFERENCES

AMINOFF, D. (1961) Methods for the quantitative estimation of N-acetyl-neuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* **81,** 384.

ANDERSEN, B. (1969) Studies on the structure of the carbohydrate moiety of rabbit γG-globulin. *Immunochem.* **6,** 739.

AVRAMEAS, S. & TERNYNCK, T. (1969) The cross-linking of proteins with glutaraldehyde and its use for preparation of immunoadsorbents. Immunochem. **6,** 53.

BOAS, N. (1953) Method for the determination of hexosamines in tissues. *J. Biol. Chem.* **204,** 553.

BUTLER, V.P., JR & VAUGHAN, J.H. (1965) The reaction of rheumatoid factor with animal gamma-globulins: Quantitative considerations. *Immunology,* **8,** 144.

CURTAIN, C., BAUMGARTEN, A. & PYE, J. (1965) Coprecipitation of some cryomacroglobulins with immunoglobulins and their fragments. *Arch. Biochem.* **112,** 37.

DAVIE, J.M. & OSTERLAND, C.K. (1968) Chemical characterization of glycopeptides from human γM-globulins. *J. exp. Med.* **128,** 699.

DISCHE, Z. & SHETTLES, L. (1948) A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. *J. Biol. Chem.* **175,** 595.

GLOVSKY, M.M. & FUDENBERG, H.H. (1969) Complement fixation activity of purified paraproteins from patients with Waldenstrom macroglobulinemia. *Clin. Res.* **17,** 353.

GYORKY, G. & HOUCK, J.C. (1965) The determination of terminal protein-bound fucose. *Canadian J. Biochem,* **43,** 1807.

HOYER, L.W., BORSOS, T., RAPP, H.J. & VANNIER, W.E. (1968) Heterogeneity of rabbit IgM antibody as detected by C'1a fixation. *J. exp. Med.* **127,** 589.

KABAT, E.A. & MAYER, M. (1961) *Experimental Immunochemistry.* 2nd Edition. Charles C. Thomas, Springfield, Illinois.

KAUZMANN, W. (1959) Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* **14,** 1.

LOSPALUTTO, J., DORWARD, B., MILLER, W. & ZIFF, M. (1962) Cryoglobulinemia based on interaction between a gamma macroglobulin and 7S gamma globulin. *Amer. J. Med.* **32,** 142.

MACKENZIE, M.R., WARNER, N.L., LINSCOTT, W.D. & FUDENBERG, H.H. (1969) Differentiation of human IgM subclasses by the ability to interact with a factor resembling the first component of complement. *J. Immunol.* **103,** 607.

MELTZER, M. & FRANKLIN, E.C. (1966) Cryoglobulinemia—A study of twenty-nine patients. γG and γM cryoglobulins and factors affecting cryoprecipitability. *Amer. J. Med.* **40,** 828.

MURAYAMA, M. (1964) A molecular mechanism of sickled erythrocyte formation. *Nature* (*Lond.*) **202,** 258.

PLOTZ, P.H., COLTEN, H. & TALAL, N.J. (1968) Mouse macroglobulin antibody to sheep erythrocytes: A non-complement-fixing type. *J. Immunol.* **100,** 752.

SAHA, A., EDWARDS, M.A., SARGENT, A.U. & ROSE, B. (1968) Mechanisms of cryoprecipitation—I. Characteristics of a human cryoglobulin. *Immunochem.* **5,** 341.

TESAR, J.T., SCHMID, F.R., GRAUER, A. & ROCHA, M.J. (1969) Heterogeneity among rheumatoid factors for complement fixation. *Arth. Rheum.* **12,** 701.

WALDESBUHL, M., ALLAN, R., MEYLAN, A. & ISLIKER, H. (1970) Anticomplementary activity of γG-immunoglobulins and of their subunits. *Immunochem.* **7,** 185.